

Bulletin of the Agricultural Chemical Society of Japan.

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The articles to be appeared in the Bulletin must be concise, supplied with experimental methods and data and understandable, without specially referring to the Japanese texts. It ought, however, not exceed four printed pages as a rule. Any longer articles may be accepted according to the decision of the Council, with or without charge for exceeding pages.

Journal of the Agr. Chem. Soc. of Japan will be published in Japanese as formerly. Those desiring the detailed information of the articles appeared in the Bulletin may look for in the Journal of the same Number or the same Volume.

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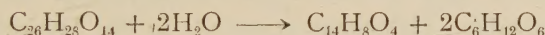
EINE NEUE SYNTHESE VON ALIZARIN. (Kondensation von Phtalsäureanhydrid mit o-Chlorphenol)

Von Munenari TANAKA.

(Aus d. Landwirt. Chem. Institut d. Universität Kyoto.)

(Eingegangen am 23. Jan. 1927.)

Das Alizarin (1.2-Dioxy-9. 10-anthrachinon) wurde im Jahre 1826 von Colin und Robiquet aus der Krappwurzel⁽¹⁾ isoliert. Es findet sich im Krapp in Gestalt eines Glykosids, der sogenannten Ruberythrinsäure, welche von Rochleder zuerst in reinem Zustand dargestellt wurde. Nach Graebe und Liebermann kommt der Ruberythrinsäure die Formel $C_{26}H_{28}O_{14}$ zu. Durch Erhitzen mit verdünnter Salzsäure zerfällt sie in Alizarin und Glykose.



Synthetisch⁽²⁾ lässt sich das Alizarin neben dem isomeren Hystazarin durch Erhitzen von Brenzkatechin mit Phtalsäureanhydrid und Schwefelsäure darstellen.

Es wurde nun beobachtet, dass sich bei der Kondensation von Phtalsäureanhydrid mit o-Chlorphenol in Schwefelsäure, drei verschiedene Körper bilden :

- I 2-Chlor-3-oxy-9.10-anthrachinon.
- II 2-Chlor-1-oxy-9.10-anthrachinon.
- III 1.2-Dioxy-9.10-anthrachinin = Alizarin.

Diese neue Synthese ist nicht nur vom theoretischen Standpunkt aus interessant, sondern auch sehr wichtig für technische Zwecke. Erhitzt man o-Chlorphenol und Phthalsäureanhydrid mit Borsäure⁽³⁾ und Schwefelsäure auf 195°, so findet man das bekannte 2-Chlor-3-Oxyanthrachinon, steigert man jedoch die Temperatur bis auf 225°, so wird die 3-ständige Hydroxylgruppe nach der 1-Stelle umgewandelt und man bekommt das unbekannte 2-Chlor-1-oxyanthrachinon. Steigert man schliesslich die Temperatur bis auf 255°, so wird das Halogenatom vollkommen in Hydroxylgruppe⁽⁴⁾

(1) Perkin and Everset : Natural Organic Colouring Matters.

(2) Liebermann : Ber. **21**, 2501 (1888)

Liebermann u. Schöller : Ber. **21**, 2503 (1888)

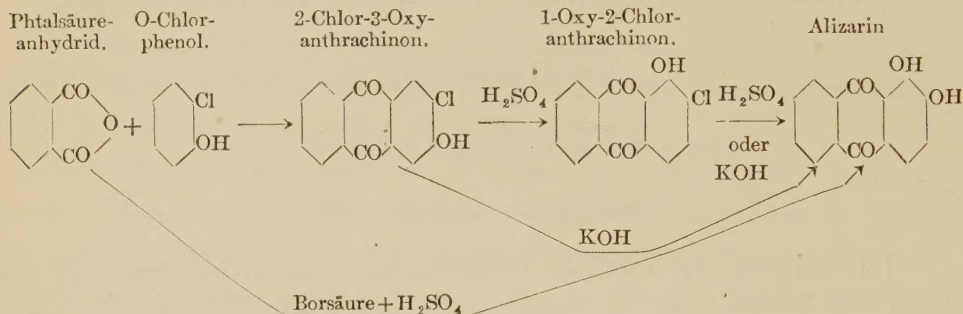
Liebermann u. Hohenemser : Ber. **35**, 1780 (1902)

Vgl. auch : Baeyer u. Caro. Ber. **7**, 972 (1874)

(3) Vergl. Ullmann : D. R. P. 282,492 u. D. R. P. 255,031

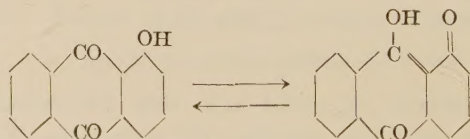
(4) Ullmann n. Conzetti : Ber. **53**, 833 (1920), By. D. R. P. 203,083.

übergeführt und eine unbeträchtliche Menge Alizarin (75 % der Theorie) gebildet.



Es hat sich ausserdem gezeigt, dass bei der Kalischmelze des 2-Chlor-3-oxyanthrachinons die 3-ständige Hydroxylgruppe zuerst nach der 1-Stelle wandert und dann das 2-ständige Halogenatom in die Hydroxylgruppe umgesetzt wird. Behandelt man 1-Oxy-2-chlor-anthrachinon mit Kali, so wird natürlich das 2-ständige Halogenatom sofort in die Hydroxylgruppe umgesetzt. Aber in beiden Fällen bildet sich sehr wenig Alizarin, da das α -ständige Halogenatom nicht so leicht in die Hydroxylgruppe umgesetzt wird wie das β -ständige Halogenatom.⁽⁵⁾

Sehr bemerkenswert ist bei dieser Umwandlung oder Umlagerung der Hydroxylgruppe dass dieselbe in der α Stelle die Verwandtschaft mit der Carboxylgruppe hat. Man kann vermuten, dass α -Oxy-anthrachinon die tautomere o-Chinoid Form besitzt.



Dies stimmt mit zwei Erfahrungen überein. Nach der von Graebe bei der Methylierung der Oxyanthrachinon gemachten Erfahrung wird die α -ständige Hydroxylgruppe weder durch Jodmethyl noch durch Methylsulfat entweder nicht oder nur sehr unvollkommen methyliert; Dimroth⁽⁶⁾ gibt an, dass in Anthrachinonderivaten die α -ständigen Hydroxylgruppen sehr viel träger acetyliert werden als die β -ständigen.

EXPERIMENTELLES.

2-Oxy-3-chlor-9. 10-anthrachinon.⁽⁷⁾

(5) Decker u. Laube : Ber. **39**, 133 (1900)

(6) Dimroth : Ber, **53**, 481 (1920)

(7) D. R. P. 148,110.

In 160g. Schwefelsäuremonohydrat werden 30g. Phtalsäureanhydrid, 15g. Borsäure und 10g. o-Chlorphenol eingetragen. Man erhitzt 1 Stunde lang auf 195°. Die Aufarbeitung erfolgt durch Eingiessen der erkalteten Schmelze in Wasser, Abfiltrieren, Auskochen mit viel Wasser und Trocknen. Die aus Eisessig und Tierkohle umkrystallisierte Substanz bildet goldgelbe Nadeln, welche bei 258° schmelzen. Die Ausbeute beträgt 3-4g.

507 mg. Sbst :	11.95 mg.	CO ₂	1.25 mg.	H ₂ O
C ₁₄ H ₇ O ₃ Cl	Ber.	C	65.0 %	H 2.71 %
	Gef.	C	64.4 %	H 2.75 %

3-Chlor-2-acetoxy-9. 10-anthrachinon.

Der Oxykörper wird in Essigsäureanhydrid zum Sieden erwärmt und 1-2 Tropfen konz. Schwefelsäure hinzugegeben. Aus Eisessig umkrystallisiert, zeigt das Acetylderivat den Schmelzpunkt 197°-200° und stellt schwach gelb gefärbte Nadeln dar.

7.12 mg. Sbst :	16.70 mg.	CO ₂	2.02 mg.	H ₂ O
C ₁₄ H ₉ Cl	Ber.	C	63.9 %	H 2.99 %
	Gef.	C	63.7 %	H 3.13 %

Umwandlung von 2-Oxy-3-chlor-9. 10-anthrachinon in Alizarin.

Man erhitzt 1g. 2-Oxy-3-chlor-9. 10-anthrachinon mit 20 g. Kali und etwas Wasser unter beständigem Schütteln bis violette Färbung eintritt. Das Reaktionsprodukt ist anfangs rot, dann dunkelgrau und schliesslich violett. Nach dem Erkalten löst man die Schmelze in Wasser, Säuert mit Salzsäure an, filtriert und trocknet. Es wird sublimiert und aus Eisessig umkrystallisiert. Schmelzpunkt 289°

3.35 mg. Sbst :	8.55 mg.	CO ₂	1.05 mg.	H ₂ O
C ₁₄ H ₈ O ₄	Ber.	C	70.0 %	H 3.30 %
	Gef.	C	69.7 %	H 3.49 %

Das Diacetylderivat schmilzt bei 179°-183°.

1-Oxyl-2-chlor-9, 10 anthrachinon.

10g. o-Chlorphenol und 32g. Phtalsäureanhydrid wurden mit 20g. Borsäure und 160g. konz. Schwefelsäure auf 225° erhitzt bis die unter Gasentwicklung eintretende Reaktion nachlässt (ca. 2 Stunden). Die Schmelze wurde mit etwas Wasser zersetzt, zur Verseifung des Borsäureesters einige Zeit gekocht und nach dem Verdünnen mit etwas Wasser der ausgeschiedene orange gefärbte 1-Oxy-2-chlorkörper filtriert und neutral gewaschen. Das Rohprodukt ergab durch Umlösen aus Eisessig mit Tierkohle 7g. reine, bei 215° schmelzende orange gefärbte Nadeln.

5.10 mg. Sbst.	12.25 mg.	CO ₂	1.20 mg.	H ₂ O
0.1015 g. Sbst :	0.0564 g.	AgCl		
C ₁₄ H ₇ O ₃ Cl	Ber.	C	65.0 %	H 2.71 % Cl 13.73 %
	Gef.	C	65.6 %	H 2.61 % Cl 13.65 %

Dieser neue Körper erwies sich leicht löslich in Alkohol, Äther, und Eisessig. Es löst sich in Alkalien mit roter Farbe. 1-Acetoxy-2-chlor-9. 10-anthrachinon stellt gelbe Nadeln (aus Eisessig) dar, Schmelzpunkt 176° – 179° .

11.20 mg.	Sbst :	26.20 mg.	CO ₂	2.80 mg.	H ₂ O
C ₁₄ H ₉ O ₄ Cl	Ber.	C	63.9 %	H	2.99 %
	Gef.	C	63.8 %	H	2.78 %

Umwandlung von 1-Oxy-2-chlor-9. 10-anthrachinon in Alizarin.

Der 1-Oxychlorkörper wird genau so behandelt wie oben sein Isomeres. Darauf entsteht Alizarin, Schmp. 289° .

5.55 mg.	Sbst :	14.15 mg.	CO ₂	1.40 mg.	H ₂ O
C ₁₄ H ₈ O ₄	Ber.	C	70.0 %	H	3.30 %
	Gef.	C	69.8 %	H	2.81 %

1. 2-Dioxy-9. 10-antorachinon.

10g. o-Chlorphenol, 32g. Phtalsäureanhydrid, 20g. Borsäure und 160g. konz. Schwefelsäure werden auf 240° erhitzt, worauf starke Gasentwicklung beginnt. Nach 1 Stunde treibt man die Temperatur auf 255° , wobei man das Eintreten der Rotfärbung bemerkt, und erhält bei dieser Temperatur so lange bis die Gasentwicklung aufhört (ca. 30 Minuten); dann wird die erkaltete Mischung, ohne zu kühlen, in kaltes Wasser gegossen, die Flüssigkeit dekantiert und die voluminöse braune Fällung nochmals mit Wasser ausgekocht, um so die unveränderten Ausgangsmaterialien, abfiltriert und getrocknet. Hierauf wird der Farbstoff mit kochendem Alkohol in Lösung gebracht und daraus umkrystallisiert. Die Ausbeute beträgt 15 g.

16.21 mg.	Sbst :	41.30 mg.	CO ₂	4.45 mg.	H ₂ O
C ₁₄ H ₈ O ₄	Ber.	C	70.0 %	H	3.30 %
	Gef.	C	69.6 %	H	3.10 %

Rote, rhomische Nadeln vom Schmelzpunkt 289° . Es sublimiert in orangeroten Nadeln. Sehr charakteristisch ist die Färbung ihrer alkalischen Lösung, in konzentrierten Zustände ist dieselbe diese purpurrot; im auffallenden Licht rein blau, durch Verdünnen geht die Farbe in blauviolett über.

ON THE DETERMINATION OF THE COLLOIDAL SUBSTANCES IN ALCOHOLIC BEVERAGES WITH THE INTERFEROMETER.

By Teizo TAKAHASHI and Toshinobu ASAI.

(Received Jan. 18th., 1927.)

Formerly one of us (T. Takahashi) and Yoshinobu Omachi (Journ, of

the Agric. Chem. Soc. of Japan. Vol 1. No. 1.) reported that the colloidal substances in alcoholic beverages were determined by means of the Löwe's interferometer, with special reference to the analytical percentage of the actual colloidal substances present. Further investigations by the same instrument have been done with respect to the colloidal substances contained in "Moto" and "Moromi" the mash of "Saké."

First we ascertained the power of adsorption of the animal charcoal (Merck's animal charcoal, extra pure). As the substances to be adsorped, such common colloidal substances, as dextrin and peptone and some non-colloidal substances, found always in the alcoholic beverages, were selected.

50c.c. of 0.1 % aqueous solution of each of these materials were added 0.5g. of animal charcoal, shaken for 30 minutes and filtered.

Of these filtrates "Colloidal Number" was found by the way as already stated. The results of the experiments were as follows :-

TABLE. No. 1.

Materials.	%(aqueous sol.)	"Drum Number,"	"Colloidal Number,"	
Dextrin. (Merck.)	0.10	100	93	} (T. Takahashi and Y. Omachi ; Journ. of the Agric. Chem. Soc. of Japan. Vol. 1. No. 1.)
Maltose. (Kahlbaum.)	0.10	103	52	
Glucose. (Merck.)	0.10	104	15	
Peptone. (Witte.)	0.10	101	94	
Alcohol.	2.0	911	70	
Saccharose.	0.10	108	46	
Glycocoll.	0.10	121	2	
Glycerin.	0.79	602	23	
Succinic acid.	0.19	157	73	

As is seen from the table, dextrin and peptone were most adsorbible and maltose, glucose, saccharose and alcohol were so to some extents. (Especially with maltose such a high "Colloidal Number" as 52, was given, as about a half that of dextrin.) Glycerin, succinic acid and glococoll were almost adsorped.

From the results above given, not only the colloidal substances but also the non-colloidal substances were adsorped in some measure, so the "Colloidal Number" which in accordance of an assumption, being absolutely concerned with the actual quantity of the colloids present in alcoholic beverages, were never been obtained so far as only this instrument gets the place. [One of

us (T. Takahashi) already proposed⁽²⁾ the application of the ultramicroscope to see whether the colloidal substances are completely adsorbed or not by the treatment. The details of these facts are to be reported before long.]

Nevertheless, we found out by this investigation a beneficial fact that the "Colloidal Number" is almost proportional to the actual amount of colloids in certain cases, at least, so in the case of "Moto" and "Moromi" of "Saké." The data are shown in the reference table (Table. No. 3).

For the determination of the colloidal substances contained in "Moto" and "Moromi" of "Saké" we selected ten periods. The dilution of the sample was respectively done according to the concentration of colloids present in each period.

In many cases they were diluted with ten times of its volume of distilled water and in another cases twenty times' dilution were taken. Animal charcoal, in various quantities, was added to 25c.c. of the diluted sample and conducted as above stated.

The "Colloidal Number" are almost equalized to hundred times of the number of grams of the actual colloids present in 100c.c. of the sample.

The following table shows the suitable quantities of animal charcoal for the determination of colloids in "Moto" and "Moromi" of "Saké", assuming dextrans and proteins were the main colloidal substances in the beverage.

TABLE. No. 2.

Stages of the sample taken.	"Futsumoto" No. 1. { In "Moromi" "Sokujio moto" No. 1. were taken in this place.				"Yamaoroshi haishi moto" No. 2.			
	Dilution.	Animal-charcoal (g. in 25c.c. of dil. sample.)	"Colloidal Number"	Protein + Dextrin (g. in 100c.c. of the sample.)	Dilution.	Animal-charcoal.	"Colloidal Number"	Protein + Dextrin.
Moto "Wakitsuki" (Beginning)	20	0.3	349	7.0796	20	0.2(?)	—	4.1616
"Moto wake" (Separation)	10	0.3	300	2.4280	10	0.3	326	3.3000
"Jyukusei" (Matured moto)	10	0.2	269	2.6172	10	0.2	267	2.5200
Before "Shiyo" (Main fermentation)	10	0.3	230	2.4132	10	0.2	252	2.3720
Moromi Before "Tome" [△]	20	0.2	196	4.1108	20	0.2	158	3.0765

(2) Journ. of the Agric. Chem. Soc. of Japan. 1925.

"Kuchiuchi"* (Main mash)	10	0.7	502	4.7307	10	0.8	541	5.7118
12 days after "Tome"	10	0.6	379	3.4687	10	0.7	452	4.5400
Before "Shiboriage" (Pressing)	10	0.3	248	2.3670	10	0.3	263	2.6246
Before "Hiire" (Pasteurisation)	10	0.1	129	1.1266	10	0.1	140	1.5588
After "Hiire"	10	0.1	127	1.2036	10	0.1	147	1.6352

△ "Tome" is the final process of the addition of the raw materials in the main fermentation.

* "Kuchiuchi" is a process of the mixing of "Moromi" mash.

The relation between the "Colloidal Numbers" and the "Drum Numbers" in the case of "Moto" and "Moromi" of "Saké" are shown in the following table :-

TABLE. No. 3.

Stage: of the sample taken.	No.	Animal charcoal (g. in 25c.c. of the dil. sample)	"Drum Number"	"Colloidal Number"	$\frac{\text{"Drum Number"}}{\text{"Colloidal Number"}}$	$\frac{\text{Alcohol + Sugar}}{\text{Dertrin + Protein}}$
"Moto" wake (Separation of "Moto" mash)	{ F. 1.	0.3	1660	300	5.53	7.60
	{ Y. 2.	0.3	1663	326	5.10	4.98
"Jyukusei"	{ F. 1.	0.2	1506	299	5.59	6.28
	{ Y. 2.	0.2	1401	267	5.24	6.01
"Kuchiuchi"	{ S. 1.	0.7	1568	502	3.12	3.09
	{ Y. 2.	0.7	1558	501	3.10	2.26
12 days after "Tome"	{ S. 1.	0.7	1363	413	3.30	5.06
	{ Y. 2.	0.7	1437	452	3.17	3.87
Before "Shiboriage" (Pressing)	{ S. 1.	0.3	1238	248	4.99	7.74
	{ Y. 2.	0.3	1294	263	4.92	7.24
Before "Hiire"	{ S. 1.	0.05	1187	94	12.62	17.29
	{ Y. 2.	0.05	1298	122	10.63	12.82
After "Hiire"	{ S. 1.	0.1	1174	127	9.24	15.62
	{ Y. 2.	0.1	1279	147	8.70	11.83

F. 1. = "Futsumoto" No. 1.

Y. 2. = "Yamaoroshi haishi moto" No. 2.

S. 1. = "Sokujiyo moto" No. 1.

Therefore, in comparison with the contents of the crystalloids such as sugars and alcohol, if the contents of the colloids were relatively larger the "Colloidal Numbers" were comparatively larger viz. there may not occur any error whatsoever caused by the adsorption of the crystalloids, and even in the reverse case the "Colloidal Numbers" show always proportional to the actual contents at least in Saké mash.

STUDIES ON ACIDS FORMED BY RHIZOPUS SPECIES.

Part III. The Formation of d-Gluconic Acid.

By Teizo TAKAHASHI and Kinichiro SAKAGUCHI.

(Received Dec. 9th., 1926.)

The occurrence of some kinds of organic acids, which are unextractive by ether, but whose lead salts are precipitable by alcohol (50–60%), in the culture medium grown with a certain species of *Rhizopus* was reported in the previous article.⁽¹⁾ To isolate and identify the acid, the lead salt yielded from about 10 liters of the medium, was treated by the current of SH_2 to set the acids. After a complete removal of excess of SH_2 , the acids were collected as calcium salts. The dry calcium salts were then treated by a small quantity of water, to get into the solution a part of these calcium salts.

To the solution thus obtained, an excess of alcohol (98%) was poured in and by this means there separated out a precipitate, which gave the characteristic colour reactions of lactic and gluconic acids.⁽²⁾

The precipitate was collected on a filter, washed several times with alcohol (98%) and at last with methyl alcohol, which dissolved very easily calcium lactate, leaving gluconate behind it. Thus we were able to isolate from the product all of the calcium gluconate, which was recrystallised from 30% alcohol in the characteristic needles.⁽³⁾ It was identified by its specific rotatory power and calcium contents as follows:—

Specific rotatory power.

Substance taken	0.3227 g.	Solvent : water.
$\alpha = + 0.37^\circ$	$l = 2 \text{ d. m.}$	$t^\circ = 17^\circ\text{C.}$
$[\alpha]_{\text{D}}^{17} = \frac{\alpha \times 100}{c \times l} = \frac{+0.37 \times 100}{3.227 \times 2} = + 5.73^\circ$		

[After Herzfeld: A. 2200, S. 345, 347, 350.

$$[\alpha]_D = + 5.^\circ 8 - + 5.^\circ 97 \quad (c = \text{Ca. } 2) \text{ and}$$

After Bertrand: A. Ch. [8], 3, 275,

$$[\alpha]_D^{20} = + 6.^\circ 13' \quad (c = 5.0)]^{(4)}$$

Contents of calcium:

Substance taken	0.2471 g. (dried at 120°C)
CaCO ₃	0.0575 g.
Ca.	0.0230 g. { found 9.31% calc. 9.37%

(1) This Journal. Vol. II. No. 20. 1926.

(2) This Journal. Vol. I. No. 14. Takahashi and Sakaguchi's article.

(3) It was about 0.4g.

(4) Beilstein. IV. Aufl. III. S. 544.

STUDIES ON ACIDS FORMED BY RHIZOPUS SPECIES. PART IV.

By Teizo TAKAHASHI and Toshinobu ASAI.

(Received. Jan. 25th., 1927.)

A. Formation of Fumaric, Succinic, Formic and Acetic acids, and of Ethyl Alcohol from Gluconic acid.

B. Formation of Succinic acid from Acetic acid.

In the first communication⁽¹⁾ on this subject the authors reported that a certain species of *Rhizopus* produced fumaric acid and ethyl alcohol not only from carbohydrates but also from tartaric acid. However, the latter acid can not be regarded as the primary cleavage product from glucose, therefore the authors studied the mechanism of the degradation of glucose more carefully, and observed, in their recent experiment,⁽²⁾ that some species of *Rhizopus* produced gluconic acid from glucose. So they investigated further to see whether gluconic acid is decomposed into simpler acids by *Rhizopus*. For this purpose two species of *Rhizopus*, i. e. Rhiz. G. 34 and G. 36 Yamazaki were cultivated in the solutions containing gluconic acid, and after being kept at 25–30° for several weeks, the culture solutions were analysed, and the presence of fumaric, succinic, formic and acetic acids as

(1) J. Agr. Chem. Soc. of Japan 2 No. 5.

(2) Ibid. 3 now in press.

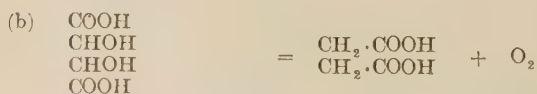
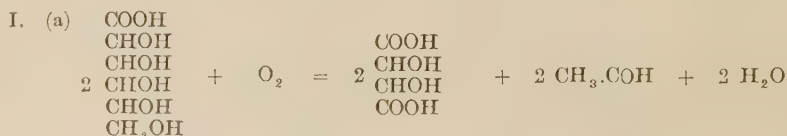
well as ethyl alcohol was positively proved. So there is no doubt that gluconic acid is the intermediate product between the above mentioned acids and glucose.

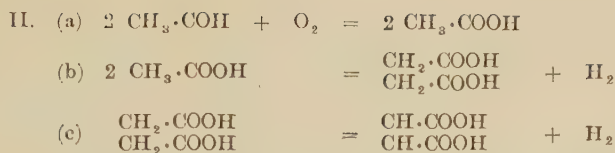
At the same time the formation of succinic acid from acetic acid, by linking two molecules of the latter together, was also confirmed.

The culture media used for this experiment were prepared by dissolving either 0.8g. peptone or 0.8g. ammonium sulphate, and 5g. calcium gluconate, a little mineral matter⁽³⁾ in 200c.c. water. The solutions were then slightly acidified with free gluconic acid in order to favour the growth of the fungus. After inoculating the fungus, the culture solutions were kept at the temperature of 25-30° for 45 to 75 days, then they were filtered and subjected to analysis with the following results :-

	I	II	III	IV	V	VI	VII	VIII
Fungi	Rhiz. G. 34.	“	“	“	Rhiz. G. 36.	“	“	“
The source of nitrogen	Peptone 0.4%	“	am-sul. 0.4%	“	Peptone 0.4%	“	Am-sul. 0.4%	“
Days of culture	45	60	48	60	55	60	70	75
Aldehyde (Schiff's reaction)	+	+	+	+	+	+	+	+
Alcohol	±	±	±	±	±	±	±	±
Acetic acid (as Ca-salt) (g.)	0.005	0.011	0.0418	0.0323	0.279	0.022	0.035	0.026
Formic acid	+	+	+	+	+	+	+	+
Fumaric acid (g.)	0.026	0.089	0.024	0.096	0.023	/	0.010	0.045
Succinic acid (g.)	/	0.016	/	0.052	0.087	0.030	0.042	0.035
Gluconic acid undecomposed	1.96	1.55	1.09	0.62	2.07	2.19	3.06	2.43

The mechanism of the formation of these organic acids from gluconic acid may most probably be represented as follows :-





For the detection and identification of the above mentioned acids, the culture solution was first distilled, and formic acid in the distillate was detected by the reducing property of a solution of potassium bichromate in nitric acid (violet blue colouration) and by that of ammoniacal mercuric chloride solution. Acetic acid was detected as acetone by the dry distillation of a small quantity of the calcium salts prepared from the distillate. The acid itself was also isolated in a pure state by the redistillation of the distillate which was previously freed from formic acid by treating with a mixture of potassium bichromate and sulphuric acid. Further, the silver salt was prepared and analysed;—

Substance taken = 0.1049 g. AgCl = 0.09 g. Ag = 0.0677 g.
 $\text{C}_2\text{H}_3\text{O}_2$ Ag. Calc. 64.64 % Ag Found 64.58 % Ag.

For the isolation of fumaric and succinic acids the culture solution was repeatedly extracted with ether, the ethereal extract was evaporated and the mixed acids were then converted into the calcium salts which were then treated with water in order to remove calcium gluconate, and the residue consisting of the mixture of calcium fumarate and succinate was taken up in ether again after acidifying with sulphuric acid. By shaking the ethereal extract with water, succinic acid went into aqueous solution, while fumaric acid remained in ethereal layer. The fumaric acid obtained by evaporating the ether melted at 285° in a sealed tube and gave positive colour reactions⁽⁴⁾ to resorcinol and β -naphthol in presence of sulphuric acid.

Number of titration:—		Substance taken	0.1133 g.
$\begin{matrix} \text{CH} \cdot \text{COOH} \\ \text{CH} \cdot \text{COOH} \end{matrix}$	n/10 NaOH	Found	19.8 c.c.
		Calc.	19.6 c.c.
Silver fumarate	$\text{Ag}_2\text{C}_4\text{H}_2\text{O}_4$	Substance taken	0.2308 g.
	AgCl 0.2003 g.	Ag 0.1508 g.	
	Ag	Found	65.34 %
		Calc.	65.43 %
Succinic acid: M. P. 183°C		(not corrected)	
Number of titration:		Substance taken	0.0443 g.
$\begin{matrix} \text{CH}_2 \cdot \text{COOH} \\ \text{CH}_2 \cdot \text{COOH} \end{matrix}$	n/10 NaOH	Found	7.0 c.c.
		Calc.	7.1 c.c.

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Silver succinate	$\text{Ag}_2\text{C}_4\text{H}_4\text{O}_4$	Substance taken	0.1124 g.
	AgCl 0.0969 g.	Ag	0.0729 g.
	Ag	Found	64.89 %
		Calc.	65.02 %

From the culture of calcium acetate medium at 25–30°C for 60 days, succinic acid identical with the above description was obtained.

A NEW BUTYL AND ISOPROPYL ALCOHOLS FERMENTATION.

By Kisaku MORIKAWA.

(Received Feb. 8th., 1927.)

The production of higher alcohols for use as solvents and in many technical developments has made great headway in the past ten years. The work of Speakman in Canada, Northrup and Fred in America and of a number of investigators in Europe has been especially worthy of mention.

It is the belief of the author of this investigation that many fermentations as yet undescribed may have technical significance and find use in industry. The present research represents one study of this character. In this I have dealt with the fermentations of Kojimash, much used in my country (Japan) and which contains large amounts of fermentable sugars.

While making some experiments in this field, I isolated certain organisms which develop and produce fermentation under aerobic conditions with the formation of what appeared to be butyl and isopropyl alcohols as the chief products of the fermentation. Continued work with pure cultures led to this investigation, in which I have described the morphology and physiology of a new organism, and have recorded the results of many studies of its fermentation reactions on various kinds of substrates.

Media—For these studies the standard media of the American Bacteriological Society were usually employed, in addition to which I have used Koji broth, Koji-agar, malt extract broth and its agar, 5% corn meal mash with calcium carbonate and other kinds of sugar broths.

STUDIES ON THE MICROORGANISMS.

Morphology—Vegetative cells appear in single rods, in pairs or in chains, having rounded ends, and enclosing granules in their cell especially in old

stages. Size of rods shows somewhat wide variability, 2 to 5 μ , majority about 3 μ in length, 0.7 to 1.5 μ , majority about 1.0 μ in width. Sporangia appear slightly swollen and with an ellipsoidal or clavated structure enclosing a spore in each cell, 2 to 5 μ , majority 3 μ in length, 1.25 to 1.5 μ , majority 1.25 μ in width. Spores are thick walled cells located at center or somewhat eccentric and having ellipsoidal structure, 1.8 to 2.5 μ , majority 2 μ in length, 0.6 to 1.25 μ , majority 1.0 μ in width. In the vegetative stage motility is very active in broth or agar cultures especially at a young stage having flagella. In old cultures it shows quite a variability of forms, swollen or somewhat clavated, and there appear difficulty stainable cells for most dyes especially methylene blue.

CULTURAL CHARACTERISTICS.

Agar stroke : With plain, Koji or malt agar, it shows good growth having echinulate, slightly raised elevation, glistening luster, smooth or little contoured surface, opaqueness, and butyrous consistency.

Gelatin stab : Liquefied medium comparatively rapidly showing good growth especially at the top.

Potato : Shows good growth with dirty white luster. Especially in the older stage it has a tendency to develop brownish colour and appears butyrous consistency of colony.

Nutrient broth : Produce good growth, strong clouding and abundant compact sediment.

Milk : Forms gas and acid, coagulating casein, afterwards digests protein very weakly. In litmus milk nearly the same character is observed without any injurious effects of litmus added. The colour of litmus is reduced gradually within a week.

Agar plate : In plain agar medium it grows rapidly appearing as circular colonies margined irregularly, smooth or rough surface, flat elevation, and shows finely curled internal structure.

Starch digestion : On starch agar plate culture no marked digestion is recognized, but with thick corn meal mush it reduces the viscosity forming gas and watery liquid.

Gas production : With arabinose, xylose, rhamnose, glucose, fructose, galactose, lactose, raffinose, inuline, mannite, dulcite, adonite, glycerin, and inosite broths, little or much gas is produced depending on the kind of substances.

Indol production : With peptone water or nutrient broth no indol is formed.

Hydrogen sulphide production : With various kinds of sodium acetate

media no hydrogen sulphide is recognized.

Nitrate reduction and ammonium production : With potassium nitrate broth or agar, nitrate is reduced into nitrite, besides ammonia is formed.

Temperature relations : Optimum 30°–40°C., minimum 10°C., maximum 50°C.

Relation to oxygen : Good growth in aerobic or partial anaerobic condition.

Relation to reaction of medium : Optimum pH 7.0 to pH 6.0.

Endo's agar stab : Bright red colouration, moderate growth along the needle track ; slight gas is formed.

Methyl red test : Strikingly positive reaction is exposed.,

The products of fermentation : Raw starch is not affected by this organism, but sugars are readily fermented yielding normal butyl and isopropyl alcohols as the chief end products. Small quantities of acetic and butyric acid are produced. The gas evolved during fermentation consists of H and CO₂, the hydrogen greatly exceeding the CO₂ in volume, and the ratio varying at different periods of the fermentation. Under semianaerobic conditions, that is, with reduced oxygen tension, small quantities of acetone are found in the distillation products.

Careful comparative studies have demonstrated that the organism is unlike any other yielding similar products which have previously been described. Most of the organisms which yield alcohols, acetone and fatty acids which have been studied up to this time may be divided into two groups.

- (1) Those which produce higher alcohols and acetone under anaerobic conditions.
- (2) Those which yield ethyl alcohol and acetone under aerobic or facultatively anaerobic conditions.

I have found no ethyl alcohol, under any of the conditions studied.

In view of these differences in morphology and physiological behavior, I believe this to be a new species and have given it the provisional name, *Bacillus Technicus*, n. sp. Prescott and Morikawa.

FURTHER STUDIES ON BIOCHEMISTRY OF THE ORGANISM.

To obtain more detailed information of physiological features, further experiments such as sugar reactions, change of gas volume which may be produced, acidity changes during incubation, and injurious effects of products on bacterial growth have been studied. All sugars tested have been consumed causing much or little gas evolution and increasing acidity. Of the pentoses, rhamnose gave little gas and alkaline reaction after seven days

fermentation, while arabinose and xylose were strongly acid and gave abundant gas formation. All the monosaccharides and disaccharides gave high acidity and much gas. The polyatomic alcohols adonite, dulcite and mannite and glycerin gave little gas. With adonite and dulcite the final reaction was alkaline, while with glycerin and mannite it was slightly acid. The alkaline reaction with adonite and dulcite is apparently the result of action on the nitrogenous constituents of the medium by which it is broken down to amines and ammonia.

Studies on the occurrence of aldehydes as an intermediate product were positive to Schiff's test in all cases. It may be supposed that the sugars are converted into aldehydes, which is instantly transformed by reduction into alcohols or by oxidation to corresponding acids.

In the early hours of the fermentation vigorous gas production and the high percentage of hydrogen in the gas evolved may be noted. This is probably due to the greater solubility of the carbon dioxide in the medium. At the intermediate period of duration the low percentage may appear, which is owing to the saturation of the solution by carbon dioxide.

An approximate ratio of hydrogen and carbon dioxide resulted 4.5 to 6.4 showing somewhat wide variation which may depend on the condition of media and the stages of fermentation tested. As to acidity change during fermentation in my process, the hydrogen ion change may show somewhat undulatory changes, that is, a rise and fall of concentration, while the titrable acidity change appears to be quite identical with that noted by others with *B. granulobacter pectinovorum*. In my investigations it was found that the titrable acidity may increase comparatively quickly, depending on the conditions, until it reaches the maximum and then it may decrease gradually to the minimum. There is not an acute increase and decrease of the acidity as in the case of *B. granulobacter pectinovorum* with corn mash.

This organism appears to be very sensitive to conditions in the medium. If there are some injurious substances in a prepared mash, the bacterial growth may be prevented. For example, with too high acidity, excessive sugar concentration or presence of a certain amount of alcohols, the bacterial growth may not appear. Although the organism here described is very sensitive to the hydrogen ion concentration at the starting point of fermentation, it may be active until about pH 4.6 with Koji mash. On the other hand, in dextrose broth this organism will grow very poorly, if at all, in pH 5.0 or a stronger acidity. At the end of fermentation, normally a fermented mash will show pH 4.4 to 4.2, or somewhat low hydrogen ion concentration, and titrable acidity 3.0 c.c. of $n/10$ sodium hydroxide solution per 10c.c. of fermented mash. In both cases, the results may be affected by the presence

of neutralizing agents. Among organic acids, which were supposed to be injurious to bacterial growth, lactic acid displayed a strongly inhibitive action, and butyric and acetic acids had weaker action in preventing growth or fermentation. This may be explainable, since the latter two acids have smaller ionization constants than the former. By an addition of about one percent of butyl alcohol before inoculation to dextrose broth, no fermentation will occur or it may be retarded, but ethyl and isopropyl alcohols may not be so harmful as butyl alcohol. The effect of their own metabolic products which are obtained from old cultures by filtering through a Berkefeld filter may be harmless in the Koji mash within certain limits.

A fermentation process has been developed, which is quite different from others now in use in which starch is converted into sugars by enzyme action of molded rice. The sugars thus produced are converted into butyl and isopropyl alcohols by the B. technicus under aerobic conditions. The consumption of sugar is most rapid in the earlier stage of fermentation, and afterwards it may show a slow decrease of the sugar content. These changes occurring somewhat in correlation with the gas production and acidity of the mash. On the contrary, the formation of alcohols may appear especially in the final stage of a fermentation. Although much unutilized sugar may remain in the fermented mash at the end of a fermentation, a good yield may be obtained in a fairly concentrated sugar mash, 12% of sugar as glucose in a mash being most favorable.

From the many experimental results it was found that 100 grams of sugar as glucose produced 30 grams of alcohol, and assuming that two mols of butyl alcohol and one mol of isopropyl alcohol may be formed from three mols of glucose, but sometimes the ratio of butyl and isopropyl alcohols showed 3:1. The yield of alcohols presumably is mostly affected by the concentration of sugar, total volume and hydrogen ion concentration of the mash. Normally 20c.c. or somewhat more alcohols may be obtained from one liter of mash. A specially high percentage yield cannot be expected since the solubility of butyl alcohol is limited, and because of the inhibiting nature of alcohols for the organisms. The determination of products such as alcohols and acids was made by testing their physical and chemical properties. Especially the alcohols were confirmed by ester formation with 3,5 dinitrobenzoic acid, and furthermore, isopropyl alcohol was tested by oxidation and then condensation with benzaldehyde. Acids were identified as toluides with paratoluidine or as paranitrobenzyl esters.

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